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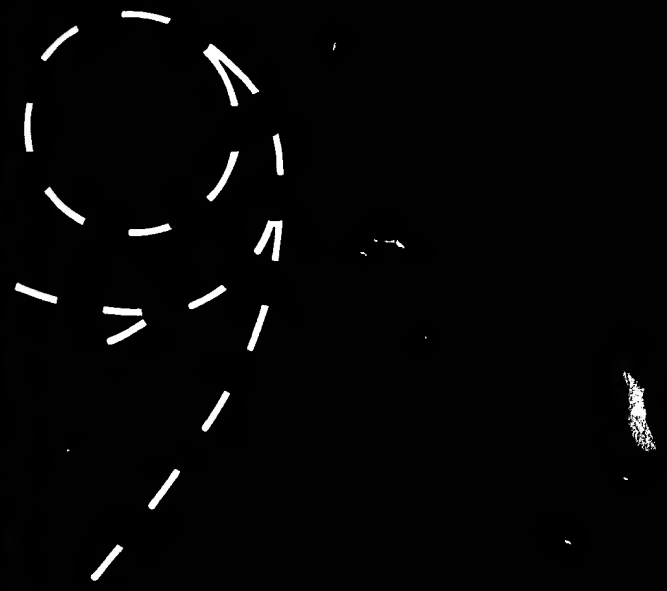

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The clonal organization of the squamous epithelium of the tongue

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Abstract. Knowledge of the kinetics and stem cell localization of the mouse lingual epithelium is largely based on studies using DNA labelling techniques. We have adopted a different approach, using histochemistry for the X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD). We have deduced clone size and morphology from studies of patch size and distribution in mice heterozygous for G6PD deficiency and from the identification of clonal enzyme loss induced in normal mice by application of a mutagen. Lingual epithelium of female mice (CBA × GPDX) heterozygous for G6PD deficiency showed multiple clearly defined patches of strong or weak enzyme activity, corresponding in intensity to the strong staining uniformly present in the normal parental strain (CBA) or to the weak staining uniformly present in the G6PD deficient parental strain (GPDX). This pattern results from the random suppression of either the paternal or the maternal X chromosome in each cell early in embryonic development, and the subsequent inheritance of X inactivation in daughter cells, giving rise to phenotypic patches each composed of one or more clones. The patch borders intersected the base of the lingual epithelium at small indentations or at the apices of connective tissue papillae; the surface intersection in some cases bisected filiform papillae. Patch width measured in tissue sections at the mid rete ridge level, showed a clear mode close to 40 μm, corresponding very closely to the mode for rete ridge width (i.e. distance between connective tissue papillae). Further evidence for clonal organization was obtained by inducing mutations in the lingual epithelium of CBA mice by topical mutagen application. A few clearly defined patches of enzyme loss were found with a mean diameter of 36 μm. Their morphology was very similar to that of patches in the heterozygous animals. We interpret these patches as clones derived from stem cells with induced somatic G6PD mutations.

We conclude that the mouse lingual epithelium is a stem cell epithelium composed of clonal units of about 40 μm diameter, based on the rete ridge structure and that both connective tissue papillae and filiform papillae occur at the junction of two or more epithelial clones.

Squamous epithelium is one of the tissues of the body that exhibits a stem cell population structure. An understanding of its clonal structure is essential not only for an insight into normal

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growth but also for studies of the early stages of carcinogenesis. The cell kinetics of the squamous epithelium of the skin and especially of the tongue have been extensively studied (Hume & Potten 1976), but its clonal structure has not been directly determined. We have used two approaches to ascertain the clonal structure of the lingual epithelium in the mouse, both of which exploit the unique nature of X-chromosome linked genes, with only a single functional allele in each mammalian cell (Lyon 1961). In female mammals the active X-chromosome in each cell is of either paternal or maternal origin. When one parent possesses a defective X-linked gene whose product can be detected histochemically, two phenotypes of cells can be distinguished. As the pattern of X-chromosome inactivation is heritable at the cellular level, clones of cells will share the same phenotype. However, as adjacent stem cells may show the same pattern of X inactivation by chance juxtaposition or because they share the same lineage, a phenotypic patch may be composed of more than one epithelial stem cell clone.

One X-linked gene whose product can be demonstrated histochemically is the housekeeping enzyme glucose-6-phosphate dehydrogenase (G6PD, EC1.1.1.49). The existence of a mouse strain (GPDX) with a level of G6PD activity only 15% of normal (Pretsch, Charles & Merkle 1988) allows the study of female mice heterozygous for normal and greatly reduced levels of G6PD activity. We have shown that enzyme histochemistry for G6PD can be applied to sues from female mice heterozygous for G6PD deficiency to show cells of two clearly distinguished phenotypes in epithelia, including thyroid and intestinal mucosa (Thomas, Williams & Williams 1988, 1989, Griffiths *et al.* 1988). We have now used this technique to study the morphology of phenotypic patches in mouse tongue epithelium, and have analysed the patch size and correlated this to the epithelial structure.

Our second approach depends upon the fact that in a normal female mammal a functioning X-chromosome gene has only a single active allele in each adult cell. A single somatic mutation may therefore interfere with the function of an X-linked gene, while mutations in both copies of a homozygous autosomal gene will be needed to lead to major changes in gene function. If the mutation occurs in a stem cell, the clone of cells maintained by that stem cell will acquire the phenotypic change dependent on the mutation. We have previously exploited this susceptibility of X-linked genes to mutation induced phenotypic change to show that colonic crypts are maintained in the adult mouse by a single stem cell, as we found loss of G6PD activity confined to single, widely scattered crypts after mutagen administration to normal mice (Griffiths *et al.* 1988). In the present study we have applied a mutagen to the tongue of normal mice, to investigate the possible development of mutations in the G6PD gene in epithelial stem cells, with consequent clonal loss of enzyme activity.

The mouse tongue is similar in its anatomy to that of most mammals, and is covered by stratified squamous epithelium with four types of specialised structures, the papillae. The filiform papillae are much the most common, they are distributed across the whole of the buccal surface of the tongue, and form backwardly projecting cones. Their anterior surfaces show 'soft' keratinization with keratohyalin granules, their posterior surfaces show 'hard keratinization with tonofilaments' (Cane & Spearman 1969, Farbman 1970). The filiform papillae are anatomically placed directly above the dermal papillae—the latter are seen in sections as prominent indentations of dermis into the basal surface of the squamous epithelium, separated by the rete ridges. When the epithelium is stripped from the dermis, and viewed with a scanning electron microscope, the basal surface is seen to be relatively smooth, apart from the deep finger-like invaginations of the dermal papillae (Kobayashi, Miyata & Ilno 1987). It is generally agreed that the stem cells lie in the basal layer of the epithelium, and that cells pass upward from the basal layer until they desquamate from the surface. Specialized columns of cells give rise to the filiform papillae (Hume & Potten 1976). The location of the stem cells, the size of

the clone of cells they maintain and the relationship of the clonal architecture to the structure of the filiform papillae need clarification. We have therefore used X-linked enzyme histochemistry to establish the clonal architecture of mouse lingual epithelium.

MATERIALS AND METHODS

Histochemistry

G6PD activity was demonstrated using a modification of a previously described technique (Thomas *et al.* 1988). Frozen sections of 5 μm thickness were incubated in a medium containing 5 mM nitroblue tetrazolium, 0.8 mM NADP, 10 mM G6P, 4mM MgC_{12}, 5 mM NaN3, 0.32 mM 1 methoxyphenazine methosulphate and 5% polyvinyl alcohol in 0.05 M tris HCl buffer at pH 6.5–7 for 12 min at 37°C. After post-fixation in 4% formal saline and washing in water, the sections were mounted in glycerine jelly. The specificity of the reaction was confirmed by the use of control sections incubated in media devoid of G6P and NADP.

Animals

Six homozygous normal female CBA mice, six homozygous female G6PD deficient (GPDX) mice and 12 heterozygous female CBA × GPDX mice were killed at 6 weeks of age with no prior treatment.

Twenty homozygous normal 6-week-old female CBA mice were divided into two groups; 10 were exposed to the oral mutagen 4-nitroquinoline-1-oxide (4NQO) applying this with a small sponge to the lingual epithelium of each mouse 3-times weekly for 8 weeks. Treatment was discontinued for 2 weeks before the animals were killed. The remaining 10 female CBA mice of the same age were used as controls. 4NQO was chosen because of its recognized mutagenicity for epithelium, and because it is a potent oral carcinogen. (Nagao & Sugimura 1976, Steidler & Reade 1984).

Tissue preparation

The tongue was dissected out immediately after death, embedded in OCT, snap frozen in isopentane cooled with dry ice, and maintained at −70°C until sectioning. Frozen sections of the tongue were cut longitudinally at 5 μm thickness on a motor-driven Bright's cryostat; they were then stained histochemically to demonstrate G6PD activity.

Analysis of sections

Stained sections were viewed under a Zeiss microscope at ×250 magnification. Sections of the heterozygous mice were also projected onto the video screen of an IBAS 2 image analyser where the anatomical and patch measurements described below were made.

RESULTS

Morphology

Untreated normal CBA mice (homozygous for normal G6PD)

Histochemistry of the lingual epithelium of all normal untreated CBA mice showed uniform strong staining with the G6PD technique throughout the squamous epithelium of the tongue except for some diminution of staining in the basal layer (Figure 1a).

GPDX mice (homozygous for deficient G6PD)

Histochemistry of the lingual epithelium of the six GPDX mice showed uniform very weak

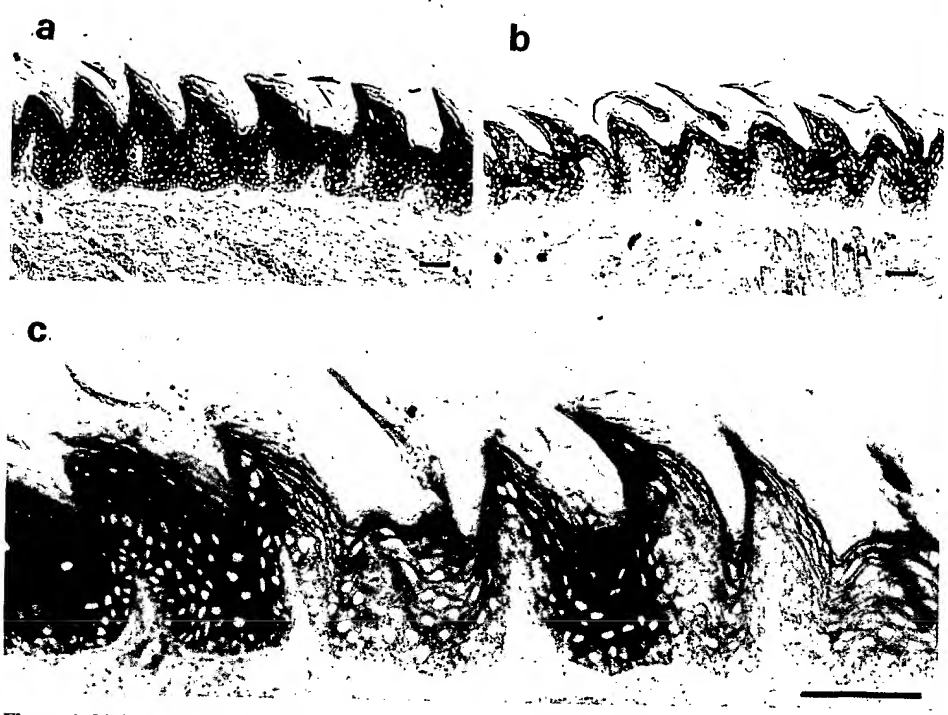

Figure 1. Uniform glucose-6-phosphate dehydrogenase (G6PD) expression in **a** homozygous normal CBA mouse and **b** homozygous G6PD deficient GPDX mouse compared with **c** phenotypic patches in lingual epithelium in a heterozygous G6PD deficient mouse (CBA × GPDX). Note the sharply defined patches of high and low enzyme activity in the heterozygote epithelium, corresponding to the levels found in the respective parental structure. Note also the intersection of patch borders with the apex of the connective tissue papilla, and the extension into the filiform papilla, dividing it into two phenotypes. G6PD histochemistry. Scale bar 50 μm.

staining with the G6PD technique throughout the squamous epithelium of the tongue (Figure 1b).

CBA × GPDX mice (heterozygous for deficient G6PD)

Histochemistry of the lingual epithelium of the 12 heterozygous mice showed two distinct levels of staining for G6PD—one level corresponding to that seen in the CBA mice and one corresponding to that seen in the GPDX mice. No intermediate levels of staining were seen. The two phenotypes formed patches with irregular but sharply defined borders running approximately vertically from the basement membrane to the surface of the epithelium (Figure 1c). The patch borders did not intersect the basement membrane randomly, the sides of the dermal papillae were spared and the borders either intersected the basement membrane at the apex of the dermal papilla or in the relatively flat epithelial/dermal junction at the base of the rete ridges. Close inspection showed that there were slight indentations in the epithelial/dermal interface which often coincided with the intersection of the patch border. The intersection of the patch borders with the oral surface of the epithelium was not marked by any anatomical landmarks except that not infrequently the border bisected a filiform papilla. Where a filiform papilla was bisected by a patch border, the division fell between the anterior and posterior columns. In a further 12 heterozygous mice the observations were repeated in sections of the

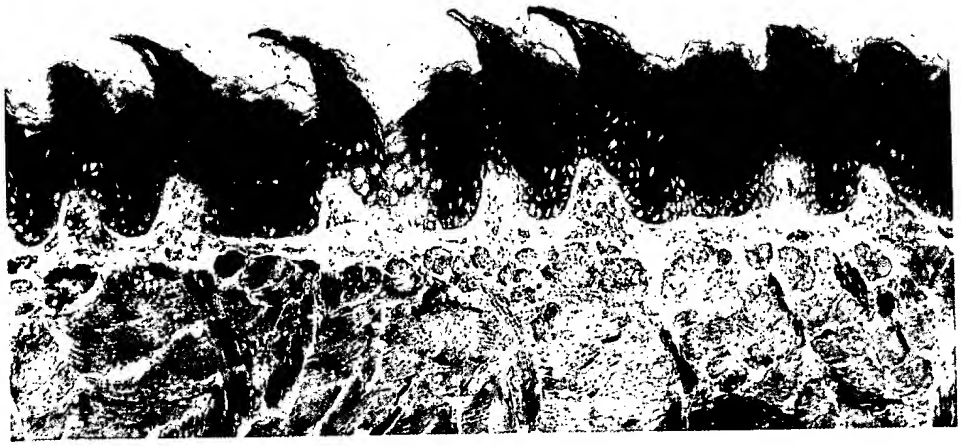

Figure 2. Mutagen-induced focal loss of glucose-6-phosphate dehydrogenase (G6PD) activity in lingual epithelium in a homozygous normal CBA mouse. The sharply defined patch of low enzyme activity closely resembles individual patches in the heterozygous G6PD-deficient mouse, and it is interpreted as a clonal loss of activity due to a mutation in a stem cell. G6PD histochemistry. Scale bar 50 μm.

tongue which had been cut transversely rather than longitudinally. The observations were unchanged except that filiform papillae were rarely composed of two phenotypes.

Carcinogen-treated CBA mice (homozygous for normal G6PD)

These animals showed no morphological abnormalities in H & E stained sections. They showed the same intensity and uniformity of epithelial staining with G6PD histochemistry as was seen in the untreated CBA mice, except that a total of seven discrete patches of very low or absent enzyme activity were seen (Figure 2). These were morphologically very similar to small low activity patches in the CBA × GPDX mice, showing the same distribution, the same sharp irregular vertical borders and the same relationship to anatomical structures.

Quantitative results

The anatomical and phenotypic patch structure for each mouse was analysed quantitatively in the sections of the heterozygous mice. A line midway between the line joining the tips of the dermal papillae and the line joining the bases of the rete ridges was defined on the image analyser. The width of each epithelial phenotypic patch defined by the histochemical stain was measured along this line. The image analyser was calibrated using a micrometer, and all measurements expressed in μm in the histological section. Where the same phenotype was shared by rete ridges separated by a connective tissue papilla, they were regarded as part of the same patch and the width of the papilla was ignored in the measurement. The width in the section plane of each individual anatomical rete ridge was measured along the same line and on the same sections; the rete ridge was defined for this purpose as the downward epithelial protrusion separated by connective tissue papillae. These measurements are illustrated diagram-

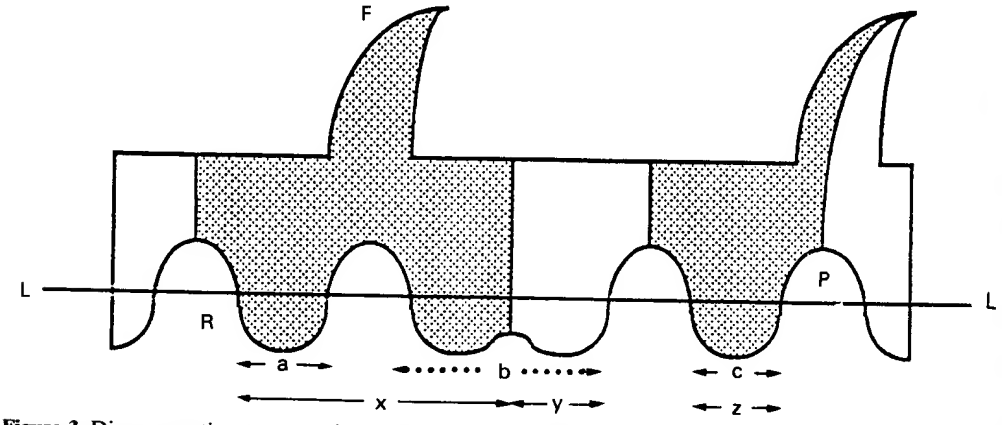

Figure 3. Diagrammatic representation of mouse lingual epithelium from a heterozygous glucose-6-phosphate dehydrogenase (G6PD)-deficient mouse. The measurements were made along the line L L midway between the apex of the connective tissue papilla (P) and the tip of the rete ridge (R). Measurements of rete ridge width are shown by the dashed lines a, b and c, and of phenotypic patch width by the continuous lines x, y and z. When a phenotypic patch included both sides of a connective tissue papilla, the width of the papilla itself was excluded from the measurement, but the patch was still regarded as a single patch. Filiform papillae are also shown (F).

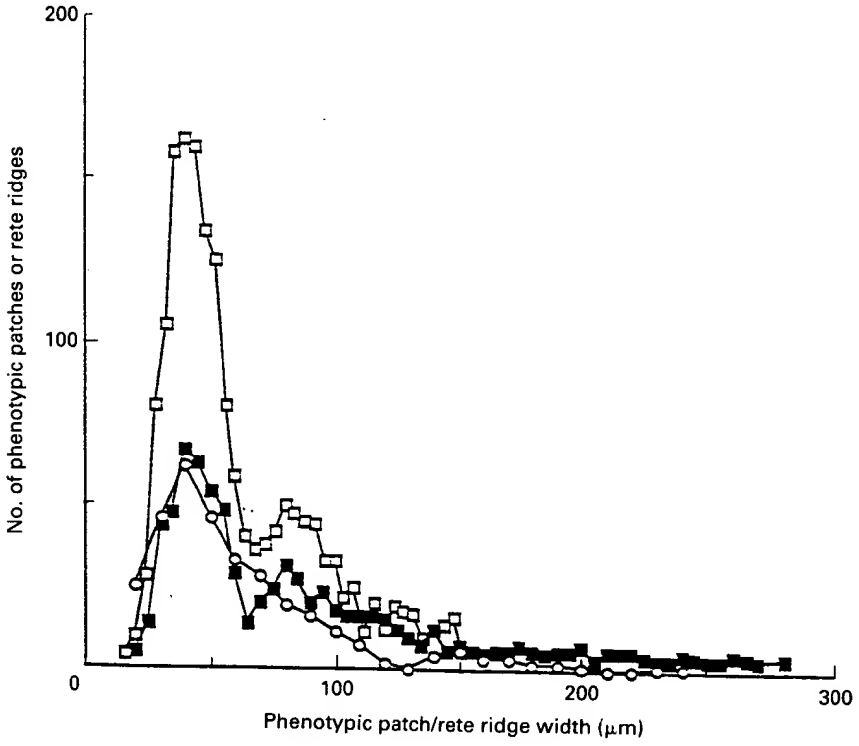

Figure 4. Frequency distribution of anatomical rete ridge widths, (■) phenotype patch widths (□) and phenotype patch width including only patches from polyphenotypic ridges (○). The primary mode for the width of both rete ridges and phenotype patches is close to 40 μm.

matically in Figure 3. A minimum of 100 contiguous phenotypic patches was measured for each mouse. The rete ridge measurements were carried out on the same areas of the same sections, approximately 180 rete ridges were measured for each animal.

Rete ridge structure

The width of each rete ridge measured as described at its mid-point was recorded over the same strip of epithelium from each CBA × GPDX mouse as was used for measuring patch size. A frequency distribution of rete ridge width was plotted (Figure 4) and found to show a clear mode at about 40 μm with a subsidiary mode at about 80 μm.

Phenotypic patch size

The width of each phenotypic patch was measured in each of the CBA × GPDX mice as described (minimum of 100 patches) and the results expressed as a frequency distribution. This showed a clear mode at about 40 μm with a subsidiary mode at about 80 μm and possibly also at 120 μm (Figure 4). To avoid the bias which could be induced by the method of measurement and the coincidence of patch and rete ridge borders, the results were also analysed after excluding monophenotypic rete ridges; the main mode of patch width remained at about 40 μm (Figure 4).

Inter-animal phenotype distribution

In the lingual epithelium studied, individual mice showed considerable variation in the total epithelial lengths showing high or low enzyme activity (Figure 5). The most uneven distribu-

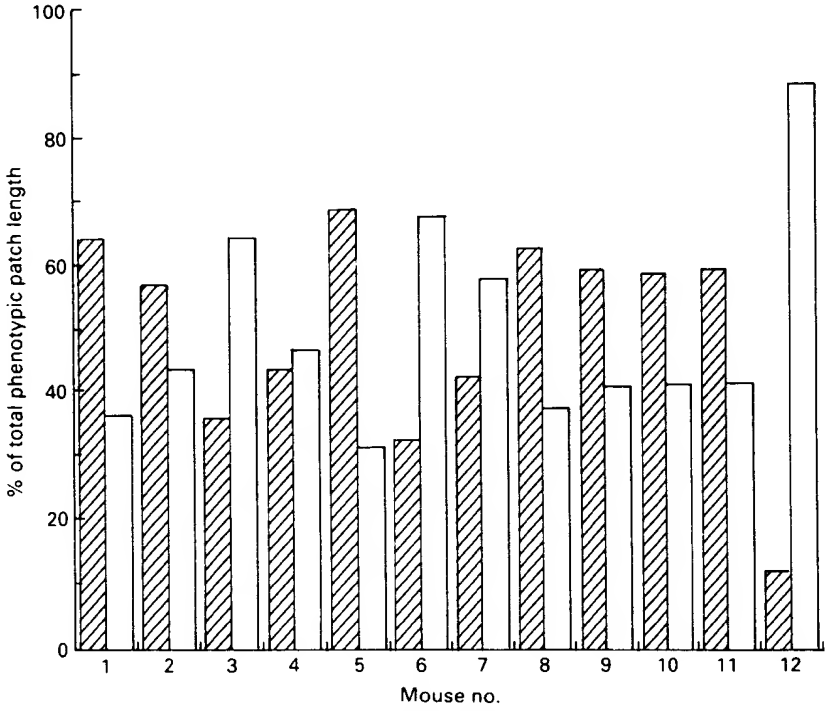

Figure 5. The accumulated length of positive (▨) and negative (□) patches from each of 12 mice, expressed as a percentage of the whole patch length measured for each mouse. When all measurements were pooled, the positive and negative percentages were close to 50%.

tion seen was in mouse no. 12, where the total length of all negative patches was 89% of the whole length of epithelium assessed. However, when the lengths of all positive patches for all mice were summed and compared with the sum of the lengths of all negative patches the result was very close to equality (51%: 49%).

Mutagen-induced patches

The width of each low enzyme activity patch in the four NQO-treated animals was measured in the same way as for the phenotypic patches. The seven patches varied in size from 12.5 to 70.5 μm, the mean width was 36 μm.

DISCUSSION

The morphological results show that the use of G6PD histochemistry and animals heterozygous for G6PD deficiency provides an excellent model for the study of the clonality of lingual epithelium. The patches of epithelium in female heterozygotes which result from the expression of the normal G6PD gene on the active X-chromosome can be clearly distinguished from the patches which result from the expression of the abnormal G6PD gene on the active X-chromosome. Phenotypic patches may of course be composed of a number of clonal units but the borders between patches must represent the borders of clonal units. Observations in the heterozygotes show that the basal cells bordering either side of a dermal papilla frequently belonged to different phenotypes, and therefore to different clones. Because of the frequency of polyclonal patches it is likely that all dermal papillae are bordered by at least two epithelial clones. The same reasoning applies to filiform papillae, where the proportion that were polyphenotypic suggests that all filiform papillae are derived from at least two clonal units. The intersection of the patch borders with the slight indentations at the dermal/epithelial interface suggests that these are markers of clonal boundaries.

To define the clonal unit more closely we have measured the width of the patches at a defined level in the epithelium and compared this with the anatomic structure of the epithelium. The coincidence of the mode for the width of the rete ridges with the mode for the width of the phenotypic patches is extremely close. To exclude any possibility that this coincidence was in part related to the coincidence of the anatomic and patch borders of rete ridges composed of one phenotype only, the assessment was repeated after the monophenotypic rete ridges were excluded. The agreement between the mode for rete ridge width and phenotype patch width when patches occupying part only of a rete ridge structure were included was still extremely close. The smallest possible phenotypic patch is the equivalent of a single coherent clone, and this result strongly suggests that a single clone in the lingual epithelium has a diameter of approximately 40 μm measured at the mid-rete ridge level.

It is possible that a clonal unit defined in this way could consist of a regular grouping of smaller functional clonal units sharing a common ancestor. To exclude this possibility we have studied a small number of widely scattered, mutagen-induced areas of enzyme loss in otherwise normal animals. The patches were sufficiently widely scattered to ensure that each patch must result from a mutation in one cell which gave rise to all the cells in the patch. There was no evidence that any ulceration or regeneration had taken place in these animals, and their epithelial architecture could not be distinguished from that of control animals. The mutated patches were very close in both size and morphology to small negative patches in the heterozygous animals. This provides very strong supportive evidence to the study in the heterozygous animals, and enables us to conclude that at the mid-rete ridge level the functional clonal unit in the mouse lingual epithelium is indeed about 40 μm in diameter.

The clonal unit of squamous epithelium in mouse skin is regarded as the epidermal proliferative unit, where one stem cell gives rise to 10 or 11 immediately surrounding basal nuclei which in turn give rise to columns of cells which ascend through the epidermis and differentiate (Potten 1974). This simple model is more difficult to apply to the mouse lingual epithelium, partly because of the presence of specialized structures such as filiform papillae which result from local variation in differentiation. Hume & Potten (1976) used both morphological and labelling studies to determine the relationship of the anatomy of filiform papillae to the stem cells that generate their cellular components. They concluded that filiform papillae represented simple modification and distortion of a number of epithelial cell columns and that there was 'a presumptive stem cell zone at the base of each dermal papilla for each of the four major columns of cells'. They located the stem cells just at the point where the basal layer of cells turns from being a horizontal layer to form the vertical border of the dermal papilla. More recent studies on mouse sulcus and gingival epithelium suggest that the cells with the greater proliferative potential are found at the deepest basal cell positions (Kellett, Hume & Potten 1989). Numerous studies have been carried out on the cell kinetics of oral epithelium, reviewed by Hume (1991). Our work has not thrown any light on the kinetics of the lingual epithelium, but does relate clonality directly to architecture. In our studies the size of the unit and its morphology as derived both from observations in heterozygous animals and the mutagen studies in homozygous animals indicate that the borders of the clonal unit in the tongue are marked in sections either by indentations in the epidermal/dermal interface, or by an indentation and the apex of the dermal papilla, or by the apices of two dermal papillae. We suggest that the stem cell lies at the base of the rete ridge. Of course the rete ridge, earlier often called a rete peg, is neither a conventional ridge nor a peg when seen in three dimensions (Kobayashi *et al.* 1987). In the dorsum of the tongue it can be regarded as an appearance in a section through an epithelial layer which has a fairly uniform thickness apart from a series of finger-like dermal invaginations—the dermal papillae. The mode of rete ridge width could therefore be regarded as the mode of the distance between dermal papillae in random sections; the clear mode of rete ridge width resulting from a regular spacing of dermal papillae. The model of the clonal structure of mouse lingual epithelium that we would derive from our observations is therefore one in which stem cells at the lowest point of rete ridges give rise to clones of about 40 μm diameter, with dermal papillae placed at the junction of clonal units.

This model has a number of resemblances to the clonal structure of the small intestine, particularly if the small intestinal epithelium is compared with the basal layer of cells of the lingual epithelium. The monoclonal crypt with its stem cell at or near the base can be compared to the monoclonal rete ridge unit with its stem cell at or near the base (Potten & Loeffler 1987, Winton & Ponder 1990) and the polyclonal villi (Ponder *et al.* 1985) can be compared to the polyclonal filiform papillae. Both filiform papillae and villi are placed at the junction of clonal units, in the small intestine the numbers of villi are much less than the numbers of crypts, and the factors that determine their location are not known. We would speculate that, in lingual epithelium and in small intestine, these factors could include population pressures where two clones meet and epithelial connective tissue interactions. The position of the basal cells in the clonal hierarchy, as the oldest basal cells are likely to be at the periphery of the clone where the specialized structures arise, may also be important.

In conclusion our observations on patch size and morphology in heterozygous G6PD deficient mice and on induced stem cell mutations in normal mice show that the clonal unit of the mouse lingual epithelium is about 40 μm in width and that filiform papillae are sometimes, and probably always, derived from more than one clone. We suggest that these observations fit best with a model in which the stem cell is at the lowest point in the mouse epithelium, giving

rise to basal cells which divide and move laterally until they meet cells of the adjacent clone. Dermal papillae are indentations located at clonal borders, where cells from adjacent clones combine to give rise in the dorsum of the tongue to filiform papillae. These observations correlate morphology and clonal structure in the tongue; both the technology and the model may help solve the problems of epithelial clonality in other tissues.

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